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Conversion of hydroxyphenylpyruvate dioxygenases into hydroxymandelate synthases by directed evolution

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Abstract Hydroxymandelate synthase (HmaS) and hydroxyphenylpyruvate dioxygenase (HppD) are non-heme iron-dependent dioxygenases, which share a common substrate and first catalytic step. The catalytic pathways then diverge to yield hydroxymandelate for secondary metabolism, or homogentisate in tyrosine catabolism. To probe the differences between these related active sites that channel a common intermediate down alternative pathways, we attempted to interconvert their activities by directed evolution. HmaS activity was readily introduced to HppD by just two amino acid changes. A parallel attempt to engineer HppD activity in HmaS was unsuccessful, suggesting that homogentisate synthesis places greater chemical and steric demands on the active site.

Keywords: Enzyme mechanism; Dioxygenase; Protein engineering; Non-proteinogenic amino acids; Hydroxyphenylglycine; Non-ribosomal peptide synthesis

1. Introduction

Hydroxyphenylpyruvate dioxygenase (HppD) catalyses the first dedicated step of tyrosine catabolism, the oxidation of 4-hydroxyphenylpyruvate (HPP) to homogentisate (HGA), with incorporation of both atoms of molecular oxygen into the product and release of CO_2 (Fig. 1) [1]. This enzyme has been a subject of research over several decades, as mutations cause type III tyrosinaemia, an inherited disorder of tyrosine metabolism, and because homogentisate is a precursor of plastoquinone and tocopherols in plants. Inhibitors of HppD are clinically and commercially important for treatment of tyrosinaemias [2] and as herbicides [3].

The closest homologue to HppD, hydroxymandelic acid synthase (HmaS), is involved in the production of glycopeptide antibiotics in *Amycolatopsis orientalis* [4,5] and at least eight other homologues have since been found in microbes with clinically important secondary metabolites. Unusual non-proteinogenic amino acids are incorporated into non-ribosomal peptides, where they contribute to the impressive chemical and structural diversity of this family of natural products [6]. HmaS catalyses the first biosynthetic step of one such amino acid, 4-hydroxyphenylglycine, by reaction of molecular oxygen with HPP to yield 4-hydroxymandelic acid (HMA) (Fig. 1).

HppD and HmaS share high homology (20–35% amino acid identity), the same substrate and the same initial catalytic step. This presents a unique opportunity to study how a common reaction intermediate is partitioned along divergent pathways by closely related active sites.

In the crystal structure of HppD from *Pseudomonas fluorescens* the catalytic Fe^{II} is coordinated by two histidines and a glutamate [7]. A hydrophobic substrate binding pocket is observed, but the orientation of substrate binding is unknown. Recently the structures of HppDs from four other organisms have become available: *Zea mays*, *Arabidopsis thaliana*, *Streptomyces avermitilis* and *Rattus norvegicus* [8–10]. The structures of *Arabidopsis thaliana* and *S. avermitilis* HppD contain inhibitors in the hydrophobic cavity, which are coordinated to Fe^{II}. Using these structural data, and the increasing number of sequenced homologues, the aim of this study was to uncover the crucial differences between HmaS and HppD active sites by interconverting their activities. Since none of the crystal structures contain substrate or products, and there is currently no structure of any HmaS, a high throughput approach was chosen for the best chance of identifying mutants with interconverted activities.

2. Materials and methods

2.1. Plasmids

The cloning of HmaS and HmaO from the antibiotic producer *Amycolatopsis orientalis* has already been described [4,15]. 4-Hydroxyphenylpyruvate dioxygenase *P. fluorescens* (HppD_PF) was amplified by PCR from *P. fluorescens* DNA, DSMZ strain 4358, and ligated in pET28a+. HppD_PF has 14 amino acid differences from the protein crystallised by Serre and coworkers [7]. The sequence has been deposited (accession number DQ364627). 4-Hydroxyphenylpyruvate dioxygenase *S. avermitilis* (HppD_SA) (accession number U11864) and HGD, a putative 1,2 homogentisate dioxygenase (accession number

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Abbreviations: HppD, 4-hydroxyphenylpyruvate dioxygenase; HppD_SA, 4-hydroxyphenylpyruvate dioxygenase *Streptomyces avermitilis*; HppD_PF, 4-hydroxyphenylpyruvate dioxygenase *Pseudomonas fluorescens*; HmaS, 4-hydroxymandelate synthase; HPP, 4-hydroxyphenylpyruvate; HGA, homogentisic acid; HMA, 4-hydroxymandelic acid

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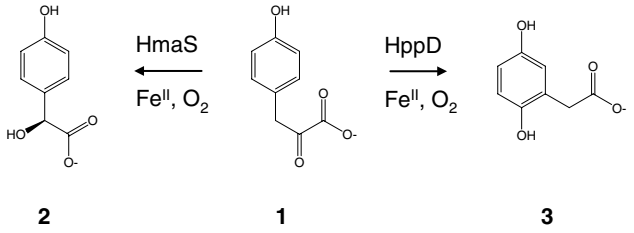

Fig. 1. Reactions of HmaS and HppD. HmaS catalyses the oxidation of 4-hydroxyphenylpyruvate (**1**) to (*S*)-4-hydroxymandelate (**2**), whereas HppD catalyses the oxidation of the same substrate to homogentisate (**3**).

AP005047, gi:29610176), were amplified by PCR from *S. avermitilis* genomic DNA and cloned in pET28a+. Primer sequences are available as supporting information.

Libraries were prepared by PCR, using oligonucleotides to encode diversified regions; a list of primers is available in the supporting information. Libraries were prepared in strain DH10B, the number of colonies was noted, then cells were scraped up to purify plasmid DNA. Libraries containing mutations at residue 335 plus one or both of 214 and 216 were prepared sequentially: residue 335 was diversified first, and this library was used as a template for PCR to introduce diversity at the other residue(s). Library quality was checked by sequencing four individual members from each library. Over 96% of library members contained full length genes with the expected diversified residues. The frequency of PCR-induced mutations was less than one per 4 kb.

2.2. Library screening in microplates

Libraries were transformed in *Escherichia coli* BL21-CodonPlus®-RP (Stratagene). Cultures were grown in 1 ml LB-tyrosine in Deepwell microplates (Fisher). LB-tyrosine was prepared from LB (1 litre) by the addition of 1 g tyrosine, dissolved in 20 ml 1 M HCl, followed by 100 ml 1 M disodium orthophosphate and 2 ml 30 mg/ml kanamycin sulphate. After 48 h growth at 30 °C, cells were harvested by centrifugation and 100 μl of the supernatant was transferred to a UV-transparent microplate (Grenier). The absorbance was measured at 280–440 nm, 10 nm intervals, using a Strectramax Plus spectrophotometer running Softmax Pro (Molecular Biosciences).

The phenotypes of the clones were classified as HmaS wild type-like ($A_{330} > 2.0$, $A_{430} < 0.1$, no visible pigmentation), HMA producer ($A_{330} > 0.3$, $A_{430} < 0.1$, no pigmentation), HppD wild type-like ($A_{330} > 2.0$, $A_{430} > 0.1$, dark pigmentation), HGA producer ($A_{330} > 0.3$, $A_{430} > 0.1$, brown pigmentation), or inactive ($A_{330} < 0.3$). Plasmids encoding genes of interest were prepared from the cell pellets by standard methods. To sample the range of amino substitutions that are tolerated by the enzymes, the genes of six wild type-like clones from each single-residue library were sequenced. Only one gene contained a PCR-induced mutation outside the active site: HmaS H106R T214V.

2.3. Overexpression and purification

All proteins were expressed in *E. coli* BL21-CodonPlus®-RP. The standard procedure was growth at 30 °C to mid-log phase followed by the addition of IPTG to 0.1 mM then 3 h expression. For HmaO, cells were grown at 37 °C to mid-log then grown at 40 °C for 6 h. Cells were lysed using lysozyme in phosphate buffer (100 mM sodium phosphate pH 8.0, 150 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme with Benzonase (Novagen)). Proteins were purified using nickel resin by standard methods, the concentration measured by the Bradford assay and the purity estimated by PAGE. Proteins were transferred to 50 mM sodium phosphate pH 7.5 for assay.

2.4. Measuring HMA and HGA production by coupled assay

HMA production was assayed spectrophotometrically by coupling to hydroxymandelate oxidase and measuring the absorbance of 4-hydroxybenzaldehyde at 330 nm. Reactions contained 20 nM–20 μM test protein and 2 μM hydroxymandelate oxidase in sodium phosphate buffer (50 mM, pH 8.0) plus 50 μM Fe^{II}, 0.5 mM ascorbic acid, 0.1 mg ml^{-1} catalase. Reactions were started by the addition of 1 mM HPP from a stock solution of 100 mM HPP in 500 mM sodium phosphate pH 8.0, and incubated at 37 °C. Reactions were determined to be tightly coupled as the rates were unchanged by increasing concentrations of hydroxymandelate oxidase. Rates quoted in Table 3 are mean values (standard error <10%) determined within the first 90 s. The extinction coefficient of 4-hydroxybenzoylformate was measured as 11 700 m^{-1} cm^{-1}.

HGA production was measured under the same conditions but using homogentisate dioxygenase as the coupling enzyme and measuring the absorption of 4-maleyl-acetoacetate at 318 nm (extinction coefficient 13 500 M^{-1} cm^{-1}).

3. Results

3.1. High-throughput activity assays

A microplate screen was developed to assay HppD and HmaS activities. Since *E. coli* lack a catabolic pathway for tyrosine, there is no endogenous production or degradation of HGA [11]. Expression of HppD in *E. coli* leads to the accumulation of HGA in the culture medium, where the spontaneous oxidation and polymerisation of HGA into ochronotic pigment provides a convenient assay [12]. Two HppDs were studied: HppD_PF from *P. fluorescens* and HppD_SA from *S. avermitilis*. Both are well expressed in *E. coli*, and the concentration of HGA in the culture supernatant reaches 0.3 g/l, whereas the detection limit for HGA is approximately 10 mg/l.

HmaS activity cannot be monitored by direct spectrophotometric assay because the absorption maxima of HPP and HMA are too similar. In cultures of *E. coli*, however, we observed slow conversion of HMA to 4-hydroxybenzaldehyde, absorption maxima at 270 and 330 nm. HMA is not an ordinary metabolite of *E. coli*, so it is likely that this conversion is catalysed by an oxidase with promiscuous substrate specificity. The product concentration in cultures overexpressing HmaS was estimated as 0.3 g l^{-1}, with a detection limit of approximately 10 mg l^{-1}. Simple analysis of culture supernatants thus enables detection of HmaS and HppD activities (Fig. 2).

3.2. Identification of crucial residues for HmaS activity

Initially five HmaS residues were chosen for mutagenesis: M199, T214, I216, I335 and Y339. These amino acids are conserved amongst all nine hydroxymandelate synthases, whereas alternative amino acids, L, P, N, F and F, are conserved in the corresponding positions in the HppD family. Furthermore, the analogous residues in HppD make up part of the active site cavity in the crystal structure of HppD_PF, and L199, P214,

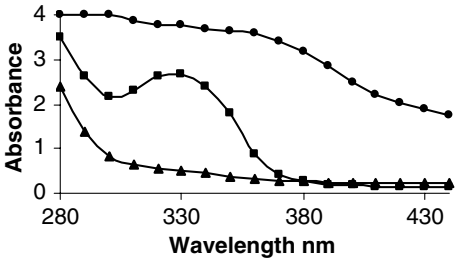

Fig. 2. High-throughput screen for HppD and HmaS activities in *E. coli*. Absorption spectra of the supernatants from *E. coli* expressing HmaS (squares), HppD (circles) or no recombinant protein (triangles).

N216 and F335 are within close proximity of the bound inhibitor NTBC in the crystal structure of HppD_SA : 5.3 Å, 3.7 Å, 4.3 Å and 3.5 Å, respectively (Fig. 3, residues numbered according to HmaS) [9].

Five libraries of genes, were constructed, each with saturation mutagenesis at a single codon. Ninety-six clones were examined from each library ($P > 0.95$ that a given codon was sampled) and their activity classified as HmaS wild type, HMA producer or inactive. The frequencies of active enzymes occurring in each library are shown in Fig. 4. Despite the complete conservation of these residues within the HmaS enzyme class, all positions were tolerant to mutation. In particular, 60% of enzymes mutated at M199 and 39% mutated at Y339 have wild type-like activity and only 18% and 52% are completely inactive. In light of their high tolerance to mutation, these two residues were not considered important for enzyme activity, and were not investigated further. T214, I216 and I335 are less tolerant to mutation, although a subset of amino acid substitutions have no effect on the final yield of HMA. To sample the range of these substitutions, six wild-type like mutants were sequenced from each library. Valine and aspartate were seen at position 214, leucine, aspartate, alanine and serine at position 216, and asparagine, valine, leucine, threonine and proline at position 335. These three positions (214, 216 and 335) were chosen for further investigation by combinatorial mutagenesis and by mutation of the corresponding residues in HppD.

3.3. Identification of critical residues for HppD activity

Libraries were constructed from HppD_SA and HppD_PF with saturation mutagenesis of P214, N216 and F335 singly and in pairs. Libraries were screened as for HmaS, revealing these HppDs to be much less tolerant to mutation than HmaS (Fig. 4). In HppD_SA library 214 and library 216 the frequency of clones with wild type-like activity is the same as the predicted frequency of wild type genes, suggesting that any amino acid substitution at these three positions disrupts enzyme function. Sequencing the clones with wild type-like activity confirmed that these clones contain proline at position 214 and asparagine at 216, whilst position 335 can tolerate hydrophobic substitutions (valine, leucine and methionine) without affecting HGA yield. Libraries of HppD_PF contained a higher frequency of clones with wild-type activity than the corresponding HppD_SA libraries. Six wild type-like mutants were from each single-residue library were sequenced, revealing a variety of substitutions: leucine and alanine at position 214 glutamine, methionine and valine at position 216, and leucine and valine at position 335.

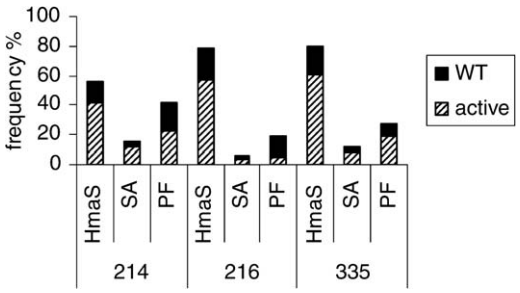

Fig. 4. Frequency of active enzymes in libraries diversified at a single residue. Solid bars show the frequency of enzymes with wild type-like activity, hashed bars represent enzymes with lower levels of activity. SA is HppD_SA and PF is HppD_PF.

3.4. Screening double HppD mutants for HMA production

No HMA producing enzymes were found amongst the single HppD mutants, but enzymes with this switched specificity

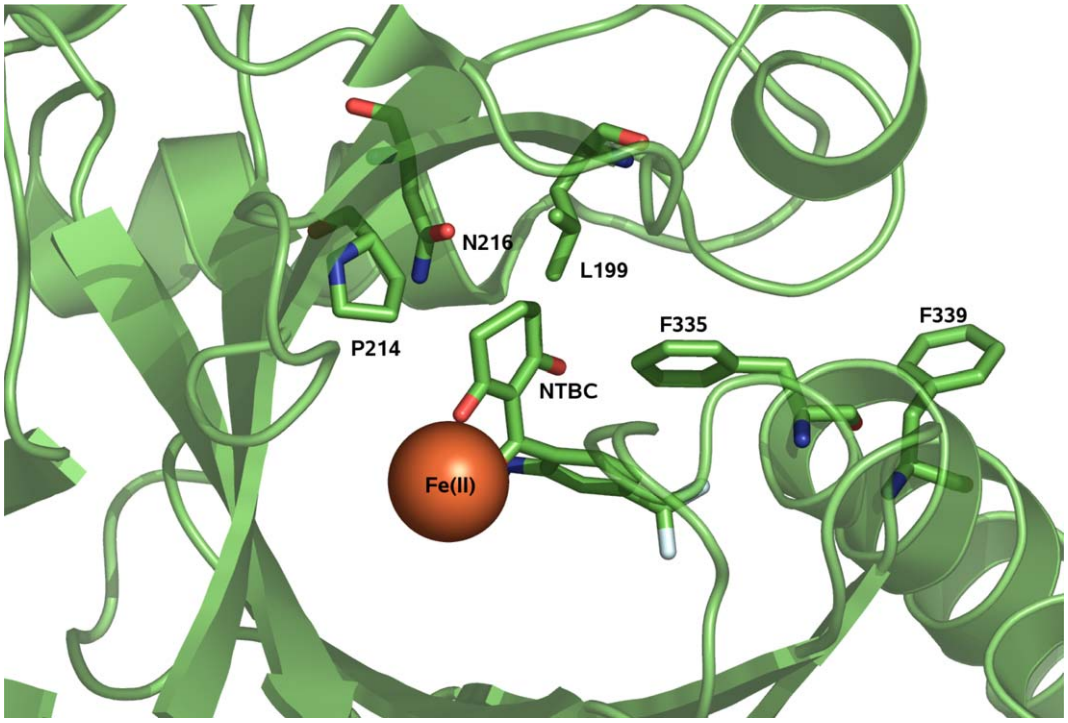

Fig. 3. Structure of *Streptomyces avermitilis* HppD with bound inhibitor NTBC.

Table 1
Frequency of HGA active mutants in HppD libraries with two randomised residues

Library	HppD_SA			HppD_PF		
	WT	HG	HM	WT	HG	HM
214 + 216	0.3	0.5	0.3	–	–	–
214 + 335	0[a]	10	2	4	9	3
216 + 335	0[a]	0.3	2	0.8	2	9

WT represents the percentage of library members with wild type levels of HGA.
HG the percentage with detectable HGA.
HM the percentage with detectable HMA.
[a]No wild type-like enzymes were found amongst 384 variants screened.

were relatively abundant in all three libraries of double mutants. From a screen of 1488 HppD mutants, 61 HMA-producing enzymes were identified. Indeed, some libraries contain a higher frequency of "reversed" HMA-producers than "wild type" HGA-producers (Table 1). A number of these double mutants with altered specificity were isolated for further study.

3.5. Screening double and triple HmaS mutants for HGA production

Analogous libraries of HmaS with mutations of pairs, or all three residues 214, 216 and 335 were constructed and screened. The frequency of active enzymes was high: 41% of clones in library 214 + 216 (12% had wild type-like yields), 20% of library 214 + 335 (10% wild type yield), 28% of library 216 + 335 (14% wild type yield) and 15% of triple mutants (6% wild type yield). No HGA-producing enzymes were detected amongst 1632 mutants screened.

3.6. Characterisation of selected clones

Sixteen HMA-producing HppD mutants were sequenced: HppD_SA variants SA1-9 and HppD_PF variants PF1-7 (Table 2). Wild type enzymes and four mutants were purified for in vitro assays. The rates of reaction were determined by spectrophotometric coupled assays: HMA was detected by enzymatic conversion to 4-hydroxybenzoylformate by hydroxymandelate oxidase, and HGA was detected by conversion to 4-maleyl-acetoacetate using homogentisate dioxygenase (Table 3 and Fig. 5). The in vitro activity assay confirmed that the wild type enzymes are extremely specific in terms of product formation, whilst demonstrating that all four mutant HppDs display HmaS activity together with a lower level of residual HppD activity.

Table 2
Sequences of HMA-producing HppD mutants

Enzyme	Residue		
	214	216	335
HppD	P	N	F
HmaS	T	I	I
SA1		F	V
SA2		L	V
SA3		F	A
SA4		F	V
SA5		F	V
SA6		F	I
SA7		L	V
SA8	T	P	
SA9	F		G
PF1		G	V
PF2		S	V
PF3		V	V
PF4		Q	I
PF5		A	V
PF6	V		T
PF7	L		A

Blank cells indicate that the residues were not mutated.

Table 3
Rates of reaction of wild type and mutant enzymes

Enzyme	Mutations	HGA production min^{-1}	HMA production min^{-1}
HmaS	Wild type	<0.02	7200
HppD_SA	Wild type	560	<0.02
HppD_PF	Wild type	232	<0.02
SA1	N216F, F335V	0.088	0.404
SA2	N216L, F335V	0.126	0.302
SA8	P214T, N216P	0.187	0.364
PF1	N216G, F335V	<0.02	0.047

Initial reaction rates were measured at 1 mM HPP.

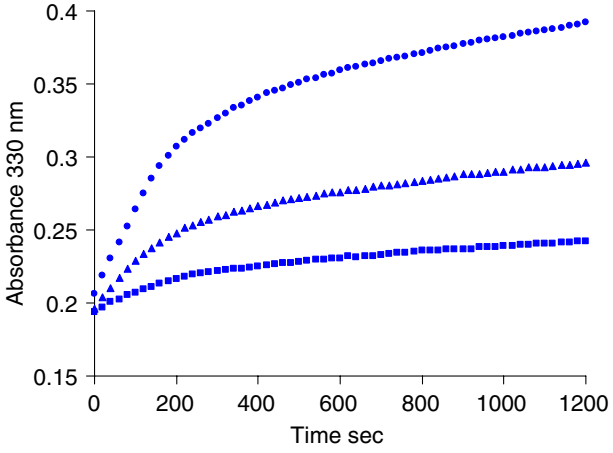

Fig. 5. HMA production by mutant SA1. Measurements taken at 1 mM HPP, with enzyme concentrations of 20 μM (circles), 10 μM (triangles) or 5 μM (squares). Substrate inhibition causes the difference between initial and steady state rates.

4. Discussion

HppD and HmaS exhibit impressive product specificity, each channelling the reactive intermediate down a single reaction pathway with at least 99.9% accuracy. In assays capable of detecting product formation at 0.02 min^{-1} (2×10^4-fold lower than the k_{cat} of HppD_SA), no side products of either enzyme could be detected. Nevertheless, if mutations are introduced to the active site of HppD then the product specificity may be switched to HMA instead of HGA. HMA product-specificity is relatively frequent amongst mutants of both HppD_SA and HppD_PF, even though HppD_PF has relatively low homology to HmaS (26% amino acid identity, compared to 34% between HppD_SA and HmaS).

Whilst this study was underway the potential of HppD mutants to catalyse HMA production was demonstrated

independently [13]. Four individual residues of HppD_SA were converted to their HmaS counterparts, and the products of these enzymes were analysed by HPLC. Two mutants, N216I and F335I, were found to yield a mixture of products which includes HMA, HGA and an unexpected product, oxepinone. The identification of oxepinone is particularly interesting, as it supports the involvement of a benzene oxide intermediate in the reaction and allowed a mechanism to be proposed [13].

Our choice of a high throughput approach allowed us to use saturation mutagenesis to effect a greater change in HppD product specificity than that achieved previously. The microplate assay is a sensitive method for detecting HGA or HMA alone, but is less sensitive for detecting the minor component of a mixture of the two molecules. For this reason, all of the HMA-producing mutants identified in this study are thought to make HMA as the major product, as confirmed by in vitro assay of four such enzymes. The HppD_SA mutants studied by Gunsior et al. were also encoded in the libraries used in this study, but they were not identified as HMA-producers during screening because they produce a greater concentration of HGA than HMA.

The interconversion of enzyme activities was attempted in both directions. In the course of screening HmaS mutants we observed a remarkable tolerance of this enzyme to mutation. Since the screen was an end-point assay, the product distribution reflects enzyme accuracy rather than efficiency. Surprisingly many HmaS mutants with substitutions at three active site residues have no reduction in HMA yield. HppD_SA, by comparison, is extremely intolerant to mutations at the analogous residues: any substitution at position 214 or 216, and most substitutions at 335, reduce or abolish the production of HGA. HppD_PF showed greater active site plasticity than HppD_SA, though it is not clear whether the difference between the enzymes reflects a general difference between gram negative and gram positive eubacterial HppDs or a difference between the particular proteins.

The different responses of HmaS and HppD to mutagenesis, and the failure to engineer HGA production in HmaS, suggest that HGA production requires an active site with a much more precise configuration than HMA production. The first step of catalysis is thought to be the same for HmaS and HppD [4,15] (Fig. 1), and this is supported by the discovery of HppD mutants that synthesise HMA (in this study and [13]). The more stringent active site requirements of HppD therefore reflect a greater level of steric control needed in the later step(s) of catalysis. Over 60 HppD mutants with changed product specificity were identified, and the mutations are not restricted to the substitution of corresponding residues from HmaS. Rather than mimicking the active site of HmaS, these mutations may block HGA formation and allow HMA to be formed as a default or side product.

Residues 214, 216 and 335 line the active site cavity of HppDs (Fig. 2). Considering the range of mutations that give rise to HMA-producing mutants (Table 3), it is clear that most substitutions preserve the hydrophobic character of the cavity, whilst perturbing the shape by the addition of one or more bulky side chains. It seems likely that similar mutations at other positions in the HppD active site, or in HppDs from other organisms, might also give rise to HMA-producing enzymes. It is interesting to note that some amino acid substitutions, such as N216V and F335V in HppD_PF, do not affect product specificity when they occur singly, but cause reversal of product specificity when they occur in combination, underlining the utility of a combinatorial approach to enzyme engineering.

The kinetics of HppD_SA have been examined in detail and it is known that this enzyme is affected by substrate inhibition [14]. Our measurements of HGA production by wild type HppD_SA were close to those previously published (k_{cat} of 7.7 s^{-1} compared to 7 s^{-1}), and we also noted that the wild type HppDs and HppD mutants suffer from substrate inhibition at substrate concentrations of 1 mM or greater (K_m 27 μM). This same inhibition is observed for HppD mutants, whether HMA or HGA production is monitored, yet HmaS itself has standard Michaelis Menten kinetics, $k_{cat} = 40\ s^{-1}$, $K_m = 149$ μM and does not display substrate inhibition (Choroba, unpublished).

Finally, the evolution of glycopeptide antibiotic gene clusters poses a difficult question: certain sugar and amino acid building blocks do not play a role in primary metabolism and their biosynthesis is encoded within the cluster. Since the biosynthetic intermediates (such as HMA) have no known function apart from further processing and incorporation into the final antibiotic product, it is hard to explain how these pathways might have evolved. The evolution of new protein functions has been proposed to arise by improvements in promiscuous enzyme activities [16]. Given their high homology, it is extremely likely that HmaS evolved from a HppD, but HppD has no detectable HmaS activity. Furthermore, HMA requires two further transformations to yield the functional building block 4-hydroxyphenylglycine. This study demonstrates the surprising ease with which the pathway might have evolved: two amino acid changes are sufficient for conversion of HppD into an HmaS, and a pre-existing promiscuous enzyme activity in *E. coli* catalyses further transformation, leading to the accumulation of millimolar concentrations of 4-hydroxybenzoylformate and reconstituting two of the three steps of 4-hydroxyphenylglycine biosynthesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.05.018.

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